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A STUDY OF THE NORMAL AND IMMUNE HEMAGGLUTININS OF THE DOMESTIC FOWL WITH RESPECT TO THEIR ORIGIN, SPECIFICITY AND IDENTITY

DISSERTATION

Submitted to the School of Hygiene and Public Health of the Johns Hopkins University in Conformity with the Requirements for the Degree of Doctor of Science in Hygiene

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Introduction.

Experimental.

1. Scope of the hemolytic and agglutinating action of chicken serum.
2. Iso-hemagglutinins in chicken serum.
3. Specificity of the various natural hemagglutinins in chicken serum.
4. The time in the life of the chick when natural hemagglutinins appear.
5. The influence of treatment with red corpuscles.
6. The identity of normal and immune hemagglutinins.

Discussion and conclusions.

Summary.

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INTRODUCTION

The discovery that the serum of one animal would hemolyze and agglutinate the red corpuscles of another was first made by Landois (1) prior to 1875, while studying blood transfusion. The fatal results that occurred when transfusions were made from one animal species to another led him to look for changes in the mixed bloods which might explain his difficulties. The clinical interest shifted to a biological one when Bordet (2) began his memorable studies of antibodies. It is to him that we owe the laying of the foundation for future work in this field.

The cause of the presence of natural hemolysins and hemagglutinins in the blood of animals is yet unsolved. There are, however, some interesting facts relating to this problem. Happ (3) found that iso-hemagglutinins and hemolysins in man developed early in childhood and that during this period the blood group of the individual becomes established. The observations of von Dungern and Hirschfeld (4), Ottenberg (5) (6) and others show that the development of these blood groups are

dependent upon factors in the germ plasm of the parents and that they are inherited in true Mendelian fashion. These two facts are important in connection with iso-antibodies and are mentioned here because of the significant light they throw on the question of the cause of normal antibodies in general.

Specificity of antibodies has been the nucleus around which the science of immunology has developed. This characteristic has been studied most, however, in connection with the behavior of immune rather than normal antibodies and least of all, the normal hemolysins and hemagglutinins.

That antibodies are transmitted from mother to offspring through the placenta and milk, is an established fact. (7) (8) It is important that the antibodies thus acquired should be excluded from a study of those antibodies of autochthonic origin. Such differentiation is not always easy in case of the mammal.

Bordet (9) was the first to produce an immune hemolysin and hemagglutinin. He studied the comparative reaction of these acquired and natural antibodies and could demonstrate no differences between the two, except quantitative ones. Since then others have studied various normal and immune antibodies, antibacterial as well as those affecting red cells, but on the whole, the results have been essentially the same as those of Bordet.

It is obvious, therefore, that the natural antibodies present many interesting questions for further investigation. There can be no doubt that a better understanding of such problems as those relating to the cause and nature of natural antibodies will throw much light on immunity reactions in general.

The present study sets forth certain facts concerning the natural and immune hemagglutinins in chicken serum with respect to their time of origin and specificity. The evidence first presented shows that the fresh serum from the adult chicken contains agglutinating antibodies for the red cells of a number of animal species. The degree of specificity of both the natural and immune hemagglutinins was determined by adsorption with the red cells from various animal species. The time of origin of a few of the hemagglutinins was determined and by taking advantage of certain facts found here, it has been possible to answer the question as to whether the natural and the corresponding immune hemagglutinins are identical.

An answer to the latter question has been facilitated by the fact that the hemagglutinins for the corpuscles tested were not present in the serum of the newly-hatched chick but arose later. This period varied from a few days to several weeks,

depending upon the species of corpuscles concerned. The latent period gave an opportunity for the production of immune agglutinins by injecting the chick with guinea pig corpuscles. A comparative study has been made of the antibody thus produced and that which normally appears later.

EXPERIMENTAL.

The technique common to all the experiments carried out in this paper is described here. Any deviations will be given with the description of the experiment in hand.

Cells. The red corpuscles were collected by cardiac puncture and immediately citrated. They were then washed four times in three or four volumes of normal salt solution, after which they were brought to whole-blood volume with normal salt solution and from this the further desired dilutions were made.

Sera. Sera from adult chickens were obtained by puncturing the jugular veins. The clot was immediately broken up and the serum and clot separated by centrifugalization. In collecting the blood from the chicks, a small needle puncture was made in

the jugular vein, and from one half to one cubic centimeter of blood was collected. The serum was pipetted from the clot after standing on ice over night. Inactivation was effected through heating the sera in a water bath at 55°C. for thirty minutes.

Titrations. The titrations were made according to one of two protocols, A or B. In protocol A, the unit for measuring serum, salt solution and cells is 0.1 c.c. and the total volume, 1.0 c.c. The materials were added to small serological tubes, as shown in protocol A, and a reading was made following incubation for one hour in a water bath at 37°C. The end titres were determined by examining microscopically a drop of the contents of a few tubes beyond the macroscopic reading. (See page 8 for protocol A)

In protocol B, the unit for measuring the serum and salt solution was a drop from a capillary pipette. The dilutions were made up as shown in protocol B; then by means of a platinum loop, hanging drops were made from each dilution plus the red cells diluted 1-5. That is, one loop of the diluted serum was placed on a cover-glass and to this was added one loop of the diluted cells. Microscopic examinations were made immediately and ten minutes after the preparations were made. (See page 7 for protocol B).

Readings. In each protocol the agglutinating titre was taken as the highest dilution of serum which gave any demonstrable clumping of the red cells.

Protocol B.

Tubes	1	2	3	4	5	6	7	8	9	10
Drops of undiluted serum	1	2	1	1	1	1 of 4*	1 of 4	3 of 7	1 of 7	0
Drops of salt solution	0	2	4	9	19	4	9	3	4	1
Titre	1	2	5	10	20	50	100	200	500	0

*Figures following the word "of" indicate the number of the tube from which the drop of diluted serum is obtained. Thus, the drop of diluted serum which is added to tube 6 was taken from tube 4. Since the serum in tube 4 had already been diluted 1-10, the serum in tube 6 is finally diluted 1-50.

Protocol A.

Tubes	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19
	1-1			1-20							1-100				1-250				
Serum	.2	.1	.05	.7	.5	.4	.3	.25	.2	.15	.7	.6	.5	.4	.2	.7	.6	.5	0
Salt Solution	.7	.8	.85	.2	.4	.5	.6	.65	.7	.75	.2	.3	.4	.5	.1	.2	.3	.4	.9
Cells(1-4)	.1	.1	.1	.1	.1	.1	.1	.1	.1	.1	.1	.1	.1	.1	.1	.1	.1	.1	.1
Titre	5	10	20	28	40	50	66	80	100	133	142	180	200	250	310	350	410	500	0

Explanation: Four preliminary dilutions of the serum to be titrated were made up as follows:

(1) 1-1. (2) 1-20, (3) 1-100, (4) 1-250, and each tube received the amount of a given dilution as indicated in the protocol. For example, tubes 1-3 inclusive received undiluted serum; tubes 4-10 inclusive received serum diluted 1-20, etc. In making the test, when no agglutinins occurred in tube 1, three other tubes were set up and into one was placed .4 c.c., another .6 c.c., and the third .8 c.c. of the undiluted serum, then the whole of each tube made up to one c.c. with salt solution and cells.

Tables. The terms "undiluted serum" when used in the tables indicates that the results recorded under that heading were from tests made with undiluted serum (hanging drops). In recording these results, the sign + (plus) indicates a positive reaction and the sign 0 (zero) indicates a negative reaction. The results recorded under the terms protocol A or B indicate that the tests were made according to the specified protocols and the results are given in figures - the figures representing the highest dilution of serum in which agglutination occurred. The upper of any two signs or figures represents the result with active serum, while the lower represents the result with inactive serum.

1. Scope of the Hemolytic and Agglutinating Action of Chicken Serum.

Bordet (10) was the first to call attention to the hemolysins and hemagglutinins of normal chicken serum, particularly for the corpuscles of the rabbit and the rat. We have made quantitative determinations of these antibodies for the corpuscles of a number of species, including the rabbit and the rat. The results are given in Table I.

Table 1. This table shows the degree of the hemolytic and agglutinating action of normal pooled adult chicken serum on the red corpuscles of other animal species.

Kind of cell	Active Serum		Inactive serum 55°C
	Hemolysis	Agglutination	Agglutination
Rabbit	28	200	250
Rat	28	200	200
Dog	10	180	180
Mouse	20	100	100
Man	28	66	66
Guinea Pig	20	50	40
Frog	10	50	40
Cat	10	40	28
Ox	10	28	28
Pigeon	0	28	20
Sheep	10	10	10
Turtle	5	5	10
Horse	2	2	5
Goat	0	0	0

Titrations were made both with active and inactive serum pooled from five to fifteen adult chickens, the agglutinating titers being determined with both active and inactive serum and the hemolytic titers with the active serum. Inactivations did not materially affect the agglutinins.

The corpuscles from fourteen different species were studied. The agglutinating titers ranged from 250 to 0. The serum was most active against rabbit cells, while goat cells were unaffected. The hemolytic titers corresponded to those of the agglutinins but they were of lower degree. The large number of species affected is most striking. Does the serum contain so many different agglutinins and hemolysins or do the same antibodies act upon the different corpuscles? This question will be considered later. Questions concerning the origin of these antibodies are also of interest.

2. Iso-Hemagglutinins in Chicken Serum.

The discovery of iso-hemagglutinins in man by Landsteiner and Shattuck (11) has stimulated attempts to demonstrate similar relations in the blood of other animal species. With few exceptions the results have been negative (12) (13).

We have made two attempts to determine whether or not

iso-hemagglutinins were present in chicken serum. The first test was carried out on thirteen adult chickens, including both sexes. The serum of each individual was tested against the cells of every other member of the flock by the hanging drop method. In this test the serum was undiluted. The cells were washed and diluted 1-4. No agglutination occurred.

The second test was made on a flock of fourteen chickens, essentially after the same manner as the foregoing. In addition to the undiluted active and inactive serum, the active serum diluted 1-5 was also tested against the cells but no agglutination occurred.

It is concluded, therefore, that chicken serum does not contain iso-hemagglutinins which can be demonstrated by the methods ordinarily used.

3. Specificity of the Natural Hemagglutinins in Chicken Serum.

The natural hemagglutinins active *for any given red cell are easily removed from chicken serum by adsorption with the given corpuscles. This is readily shown by titrating the adsorbed serum with the corpuscles in question. If the adsorbed serum is titrated against corpuscles from a species different from the one used in the adsorption, the agglutinins for the different corpuscles may or may not be found to have disappeared.

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*Explanatory Note: In studying the hemagglutinins in chicken serum, there arose the need for a new term to denote a particular action of these antibodies for the corpuscles of a given animal species. For example, the normal serum would sometimes agglutinate rabbit corpuscles in a dilution of 1-200. It was also found that not all these antibodies which agglutinated the rabbit cells were specific but, as shown by adsorption tests, some of the antibodies would agglutinate other kinds of cells such as those of the guinea pig, rat, etc. Then it was obvious that one could not speak of these anti-rabbit hemagglutinins as specific nor were they entirely non-specific. They were, in truth, specific plus non-specific anti-rabbit hemagglutinins. The same need for a term to fit this particular kind of case came up in connection with the action of other hemagglutinins. The word "active" will be used to express this reaction. That is, in speaking of all the hemagglutinins which agglutinate the red corpuscles of the rabbit, we say, "the hemagglutinins active for the red corpuscles of the rabbit". The same is applied to anti-rat, anti-guinea pig, and other hemagglutinins. In this paper, the term "active" used in this connection need not conflict with the meaning of the term active when applied to serum to indicate that it contains complement.

The result depends upon the kind of corpuscles used in the test. The results from a series of adsorption experiments with the red corpuscles from five different animal species show that chicken serum does not contain agglutinins which react separately for each of these kind of cells, nor does the agglutinating substance act as a single unit. The experimental objective here concerned was to determine whether different agglutinating antibodies were present, or if the agglutinating substance does act as a unit, how complex its binding properties are.

The percentage of agglutinins active for one corpuscle, which is adsorbed by another, was measured by comparing titrations made before and after absorption. For convenience in working, the kinds of cells were divided into groups of threes and the specificity of the agglutinins determined for the different cells of each group.

The grouping of cells is as follows:

Group One : Guinea Pig, rabbit and rat.

Group Two : Rat, goat and sheep.

Group Three : Rabbit, goat and sheep.

Group Four : Guinea Pig, goat and sheep.

The various groups of cells were tested in the same way. Using cells of group one as a type, the procedure was as follows:

a. Pre-adsorption Titrations. - The agglutinating titre of pooled inactive chicken serum was determined for the red cells of the guinea pig, rabbit and rat, according to protocol A. These are referred to as the normal or pre-adsorbed titres and it is with these that the post-adsorbed titres are compared.

b. Adsorption Process. - From the same lot of serum from which the pre-adsorbed titrations were made, 5 c.c. of undiluted serum was added to concentrated guinea pig cells (concentrated by centrifugalization from 5 c.c. of blood), the mixture being thoroughly stirred and then incubated for one hour. The cells were then thrown out by centrifugalization and the serum removed. A similar preparation was made with rabbit cells and also with rat cells.

c. Post-adsorption Titrations. - Each of the three adsorbed sera obtained under process[b] was now titrated against the red corpuscles of the guinea pig, rabbit and rat according to protocol A. The result of this test is given in Table II. A study of this Table brings out the following points:

Table II This table shows the percentage of hemagglutinins removed from pooled chicken serum when adsorbed by the red corpuscles of the guinea pig, rabbit and rat, as shown by the subsequent titrations with corpuscles from the same species.

Kind of cell used in adsorbing	Guinea pig			Rabbit			Rat		
Kind of cell used in titrating	Guinea Pig	Rabbit	Rat	Guinea Pig	Rabbit	Rat	Guinea Pig	Rabbit	Rat
Pre-adsorbed titres	20	200	250						
Post-adsorbed titres	0	50	50	0	0	50	0	40	0
Titres after second adsorbing	0	50	40	0	0	40	0	28	0
Percentage of agglutinins removed	100	75	80	100	100	80	100	80	100

(1) All of the agglutinins active for guinea pig corpuscles are removed by either rabbit or rat corpuscles.

(2) Approximately three-fourths of the agglutinins active for rabbit corpuscles are removed by either guinea pig or rat corpuscles.

(3) Approximately three-fourths of the agglutinins active for rat corpuscles are removed by either rabbit or guinea pig corpuscles.

Apparently, only about twenty-five per cent of the anti-rat agglutinins and twenty-five per cent of the anti-rabbit agglutinins are adsorbed specifically. All of the agglutinins which are active for guinea pig cells, about three-fourths of the agglutinins which are active for rabbit cells and about the same proportion of the agglutinins active for rat cells, can be adsorbed equally as well by any one of these three kinds of cells.

Another adsorption experiment was performed. This second experiment was carried out in the same way as the one just described above, with the exception that after the undiluted serum had been adsorbed, and before it was titrated, it was again adsorbed with fresh cells of the same kind and in the same amounts as were used in the first adsorption. This was to answer the

question as to whether the red cells were removing the agglutinins in some mechanical way, or became saturated before all the agglutinins had been taken up. If either or both of these conditions were true, a second adsorption of the serum with fresh cells would remove the remaining anti-bodies active for a particular kind of cell. The results of this experiment are also shown in Table II. It is apparent that practically no antibodies were removed by the second adsorption.

The question arises as to whether the agglutinins which are adsorbed non-specifically are identical or only apparently so, because the degree of non-specificity chanced to be the same. A simple test showed the former condition to be the true one. This was done by carrying out another adsorption experiment, as follows: In addition to adsorbing one lot of serum with guinea pig cells, a second with rabbit cells and a third with rat cells, a fourth lot was adsorbed with both guinea pig and rabbit cells, a fifth lot with both guinea pig and rat cells, and a sixth lot with both rabbit and rat cells. Each lot of the adsorbed serum was then titrated against each of the three kinds of cells and a comparison of pre-adsorbed post-adsorbed, and post double-adsorbed titres was made. A

record of these titres is shown in Table III. From the record it can be seen that the guinea pig and rabbit cells together removed practically no more agglutinins for rat cells than each alone removed. The same is true in case of the removal of agglutinins for rabbit cells by guinea pig and rat corpuscles. The results suggest that the red corpuscles of the guinea pig, rabbit and rat are, in this case, removing identical antibodies. Rabbit cells, in addition to removing these identical antibodies, will, at the same time, remove their specific agglutinins. This is also true of the rat corpuscles.

Chicken serum was now tested against cells of groups two, three and four, and the results recorded in Tables IV, V, and VI, respectively. The same thing happened here as with the cells of group one. When a specimen of serum was adsorbed with any two kinds of cells, little more, if any, of the agglutinins active for a third kind of corpuscle were removed than when the serum was adsorbed with the one having the higher adsorption percentage. It is an interesting fact, that although goat cells are not agglutinated, they are capable of adsorbing the agglutinins active for other cells, namely, those of the rabbit and the guinea pig. Also, when sheep cells show no agglutinating titres, they nevertheless take up antibodies active for other cells.

Table III. This table shows the percentage of hemagglutinins removed from the pooled chicken serum when adsorbed by any one or two of the red corpuscles of the guinea pig, rabbit and rat as shown by subsequent titrations with corpuscles from the same species.

Group One

Kind of cells used in adsorbing	Rabbit			Guinea pig			Rat			Rabbit and Guinea pig			Rabbit and rat			Rat and Guinea pig		
Kind of cells used in titrating	Rabbit	Guinea Pig	Rat	Rabbit	Guinea Pig	Rat	Rabbit	Guinea Pig	Rat	Rabbit	Guinea Pig	Rat	Rabbit	Guinea Pig	Rat	Rabbit	Guinea Pig	Rat
Pre-adsorbed titres	133	40	410															
Post-adsorbed titres	0	0	80	40	0	66	50	0	0	0	0	80	0	0	0	28	0	0
Percentage of agglutinins removed	100	100	80	65	100	81	60	100	100	100	100	80	100	100	100	79	100	100

Table IV. This table shows the percentage of hemagglutinins removed from pooled chicken serum when adsorbed by any one or two of the red corpuscles of the rat, goat and sheep as shown by subsequent titrations with corpuscles from the same species.

Group Two

Kind of cells used in adsorbing	Rat			Goat			Sheep			Rat and Goat			Rat and Sheep			Goat and Sheep		
Kind of cells used in titrating	Rat	Goat	Sheep	Rat	Goat	Sheep	Rat	Goat	Sheep	Rat	Goat	Sheep	Rat	Goat	Sheep	Rat	Goat	Sheep
Pre-adsorbed titres	310	0	0															
Post-adsorbed titres	0	0	0	310	0	0	310	0	0	0	0	0	0	0	0	310	0	0
Percentage of agglutinins removed	100			0			0			100			100			0		

Table V. This table shows the percentage of hemagglutinins removed from pooled chicken serum when adsorbed by any one or two of the red corpuscles of the rabbit, goat and sheep as shown by subsequent titrations with corpuscles from the same species.

Group Three

Kind of cells used in adsorbing	Rabbit			Goat			Sheep			Rabbit and Goat			Rabbit and Sheep			Goat and Sheep		
Kind of cells used in titrating	Rabbit	Goat	Sheep	Rabbit	Goat	Sheep	Rabbit	Goat	Sheep	Rabbit	Goat	Sheep	Rabbit	Goat	Sheep	Rabbit	Goat	Sheep
Pre-adsorbed titres	410	0	10															
Post-adsorbed titres	0	0	0	180	0	0	250	0	0	0	0	0	0	0	0	133	0	0
Percentage of agglutinins removed	100		100	56		100	39		100	100		100	100		100	67		100

Table VI. This table shows the percentage of hemagglutinins removed from pooled chicken serum when adsorbed by any one or two of the red corpuscles of the guinea pig, goat and sheep as shown by subsequent titrations with corpuscles from the same species.

Group Four

Kind of cells used in adsorbing	Guinea pig			Goat			Sheep			Guinea pig and goat			Guinea pig and sheep			Goat and sheep		
Kind of cells used in titrating	Guinea pig	Goat	Sheep	Guinea pig	Goat	Sheep	Guinea pig	Goat	Sheep	Guinea pig	Goat	Sheep	Guinea pig	Goat	Sheep	Guinea pig	Goat	Sheep
Pre-adsorbed titres	28	0	10															
Post-adsorbed titres	0	0	0	20	0	0	1	0	0	0	0	0	0	0	0	1	0	0
Percentage of agglutinins removed	100		100	28		100	96		100	100		100	100		100	96		100

(See Table V). Apparently, goat cells do not adsorb agglutinins active for rat cells.

Of the five agglutinins studied, none active for a given kind of cell shows absolute specificity when measured by adsorption tests. Only two, those active for rabbit and rat cells showed partial specificity throughout all the tests. Anti-guinea pig agglutinins showed partial specificity when adsorbed with goat and sheep cells.

It is suggested from the behavior of guinea pig cells in their adsorption of antibodies that, like the corpuscles of the goat and sheep, they adsorb hemagglutinins which do not enter into their agglutinating reaction. Thus we find that one unit of guinea pig cells adsorbed from two to eight units of agglutinins active for other cells (rabbit and rat), but the guinea pig cells were not agglutinated. Sheep and goat cells each have adsorbed five units of anti-rabbit agglutinins without being agglutinated. The fact that the goat cells remain immune to the action of any of these antibodies, leads us to think that the guinea pig cells are behaving similarly. It might be that several units of anti-rabbit or anti-rat hemagglutinins are required to make one unit of anti-guinea pig hemagglutinins. Should this be true, then variations in the agglutinating titres for rabbit, rat and guinea

Table VII. This table shows a summary of the percentages of hemagglutinins removed from pooled chicken serum when adsorbed by the red corpuscles of the rat, rabbit, guinea pig, sheep and goat.

		Kind of cell used in adsorbing				
		Rat	Rabbit	Guinea pig	Sheep	Goat
Kind	Rat	100	80	80	0	0
of	Rabbit	60	100	65	40	60
Cells used	Guinea pig	100	100	100	96	28
in	Sheep	100	100	100	100	100
titrating	Goat	0	0	0	0	0

pig cells should run parallel. That this does not always happen can be seen by comparing the titres for these cells shown in Tables II and III.

A summary of the per cent of hemagglutinins for one kind of corpuscle which are removed by another kind is given in Table VII.

4. The Time in the Life of the Chick when Normal Hemagglutinins Appear.

A series of tests, made with the serum from seventeen chicks, show that the hemagglutinins which exist normally in the blood of the adult fowl are not present at hatching, but appear at a definite time afterwards. These tests were made upon two lots of chicks, one a flock of eight pure Plymouth Rocks and another of nine pure White Leghorns from an incubator hatch. Five tests were made on the Plymouth Rocks; the first on the twenty-ninth, the last on the seventy-seventh day after hatching. Eight tests were made on the White Leghorns; the first on the tenth day and the last on the fifty-eighth day after hatching. The first tests were made to determine the presence or absence of the antibodies in the active and inactive serum. This was done by making hanging drops with the undiluted serum and the red cells. When the chicks were old enough to withstand the loss of larger

Table VIII. This table shows the age at which hemagglutinins began to appear in the blood stream of nine White Leghorn Chicks.

Method Used	Undiluted serum																											
	Protocol B																											
Age of chick in days	10			16			23			30			30			38			44			51			58		
Kind of cell used	Guinea Pig	Rabbit	Rat	Guinea Pig	Rabbit	Rat	Guinea Pig	Rabbit	Rat	Guinea Pig	Rabbit	Rat	Guinea Pig	Rabbit	Rat	Guinea Pig	Rabbit	Rat	Guinea Pig	Rabbit	Rat	Guinea Pig	Rabbit	Rat	Guinea Pig	Rabbit	Rat
1	0	0	0	0	0	0	0	+	+	+	+	+															
	0	0	0	0	0	0	0	+	+	+	+	†															
2	0	0	0	0	0	0	0	0	0																		
	0	0	0	0	0	0	0	0	0																		
3	0			0	0	0	0	0	0	0	0	+				0	5	1	0	20	2	0	50	200	1	20	50
	0			0	0	0	0	0	0	0	0	†	0	1	1	0	5	1	0	200	2	0			2	100	200
4	0	0	0	0	0	+	0	+	+	0	+	+				1	50	5	2	200	100	2	50	20	1	20	500
	0	0	0	0	0	†	0	+	+	0	+	+	0	10	5	0	50	5	5	500	10	2			1	600	600
5	0	0	0	0	0	+																					
	0	0	0	0	0	†																					
6	0	0	0	0	0	0	0	0	0	0	0	+				0	10	0	2	200	5	1	10	50	0	100	600
	0	0	0	0	0	0	0	0	0	0	0	†	0	0		0	10	1	1	200	2	5			0	200	500
7	0	0	0	0	0	0	0	0	+	0	+	+				0	10	2	2	200	20	2	50	20	5	100	600
	0	0	0	0	0	0	0	0	†	0	+	+	0	2	5	0	10	2	5	500	20	5			5	100	600
8	0	0	0	0	+	0	0	+	+	0	0	†				1	5	1	2	500	10	1	100	50	0	500	600
	0	0	0	0	+	+	0	0	+	0	0	†	0	0	1	2	10	1	2	500	10	2			1	500	600
9	0	0	0	0	0	0	0	0	†	0	0	+	0	0	10												
	0	0	0	0	0	0	0	0	†	0	0	†															

Table IX. This table shows the age at which hemagglutinins began to appear in the blood stream of eight Plymouth Rock Chicks.

Method used	Undiluted Serum					Protocol A			
Age of chick in days	29	36		56	70	77	77		
Kind of cell used	Guinea Pig	Rabbit	Rat	Guinea Pig	Rabbit	Rat	Rabbit	Rabbit	Guinea Pig	Rat
1	0	0	0	0	± ±	± ±	133		0	28
2	0	0	0	0	0	0				
	0	0	0	0	0	+		133	0	100
3	0	0	0	+	+	+				
	0	0	0	+	+	+	310	500	10	133
4	0	+	0	0	+	+				
	0	+	+	0	+	+	310	133	10	133
5	0	0	0	+	+	+				
	0	+	0	0	+	+	310	200	5	133
6	0	0	0	0	0	0				
	0	0	0	0	0	0				
7	0	0	0	0	+	+				
	0	0	0	+	+	+	350	180	20	100
8	0	0	0	+	+	+				
	0	0	0	+	+	+	500	142	10	50

amounts of blood, the exact titres were determined with both the active and inactive sera, according to protocols A or B. The results from these tests are shown in Tables VIII and IX.

The evidence presented in these tables demonstrates that the hemagglutinins existing in the serum of the adult chicken for the red corpuscles of the guinea pig, the rabbit and the rat appear between the second and fifth week after hatching. Those active for rabbit and rat cells appear first, about the sixteenth day, and those active for guinea pig cells appear about three weeks later. The amount of hemagglutinins gradually increases over a period of several days until the adult titre is reached. This period is for rabbit cells about twenty days, for rat cells about thirty days. The time cannot be determined from these records for guinea pig cells, as the tests did not continue until the titre for these cells had reached that of the adult. It is evident, however, that the time is more than thirty days.

5. The Effect of Treating Chicks with Red Corpuscles.

It has been demonstrated (part 4) that the agglutinins for the red corpuscles of the guinea pig do not appear in the serum of the chick until between the fourth and fifth week after hatching. This is about three weeks after the appearance of

the agglutinins for the red corpuscles of the rabbit and the rat. With this fact in mind, the question was raised as to the possibility of stimulating the anti-guinea pig agglutinins to appear earlier by the injection of the specific antigen. The question was put to test in the following experiments:

a. Anti-guinea pig experiment. - Seven pure White Leghorn chicks were each given intraperitoneally an injection of 0.5 c.c. of a 1-5 dilution of guinea pig corpuscles. Two chicks were kept for controls. The injections were repeated on the fourth, sixth and ninth days after hatching, and the first test for agglutinins was made six days following the last injection. The chicks were by this time fifteen days old. Two subsequent tests were made. The results from all the tests are recorded in Table X.

From the results shown in the Table (X), it can be seen that the agglutinins active for guinea pig cells have appeared fifteen days earlier in the chicks which received the injections than in normal chicks (see part 4, Table IX). The controls, chicks Nos. 8 and 9, in this test, had not developed the antibodies when the last test was made twenty-seven days after hatching. It is noted that out of seven treated birds, three (chicks Nos. 1, 5, and 6) had reached titres several times higher than the titre shown by the adult for guinea pig cells. Two (Nos. 2 and7)

Table X. This table shows the effect of injections of chicks with red corpuscles of the guinea pig with respect to time of origin of the hemagglutinins.

Method Used		Protocol B								
Age of chick in days		15			20			27		
Kind of cell used		Guinea Pig	Rabbit	Rat	Guinea Pig	Rabbit	Rat	Guinea Pig	Rabbit	Rat
No. of chicks										
Treated Chicks	1	500	0	0	dead					
	2	20	0	0	20	0	0	2	2	0
	3	0	0	0	0	0	0	0	0	0
	4	2	0	0	dead					
	5	200	0	0	5	0	0	5	500	500
	6	500	0	0	10	0	0	10	10	2
	7	20	0	0	dead					
Controls	8	0	0	0				0	500	500
	9	0	0	0				0	10	10

showed titres comparable to that of the adult, while one (No. 4) showed a low titre, and another, (No. 3) showed no agglutinins. It is significant that when the injections are stopped, the titre gradually falls. It is also significant that these antibodies do not agglutinate rabbit or rat cells, neither do the agglutinins for these latter cells originate any earlier in the treated animals.

b. Anti-rabbit experiment. - A series of chicks treated with red cells from the rabbit gave results which were surprisingly different from those obtained with the anti-guinea pig series. For this experiment a hatch of thirteen chicks was used. None of these was of pure stock, but a mixture of White and Brown Leghorn, Plymouth Rock and Wyandote. When they were twenty-four hours old, nine of them (Nos. 1 - 9 inclusive, Table XI) were each given an intraperitoneal injection of 0.5 c.c. of rabbit cells, 1-5 dilution. The other four were kept for controls (Nos. 10 - 13 inclusive, Table XI). These injections were repeated on the fourth and sixth days after hatching. When the chicks were eight days old (four days following the last injection) their sera were tested for agglutinins. Two subsequent tests were made. The results are recorded in Table XI. It is readily seen from the Table that the injection of the chicks with rabbit corpuscles has not hastened the time of appearance of

Table XI. This table shows the effect of injecting chicks with red corpuscles of the rabbit with respect to the time of origin of hemagglutinins.

Method Used		Undiluted Serum								Protocol B			
Age in days		8			13			21			27		
	Kind of cell used	Guinea Pig	Rab-Rat bit		Guinea Pig	Rab-Rat bit		Guinea Pig	Rab-Rat bit		Guinea Pig	Rab-Rat bit	
	No. of chicks												
Treated Chicks	1	0	0	0	0	0	0	0	0	0	0	2	500
	2	0	0	0	0	0	0	0	0	500	0	2	200
	3	0	0	0	0	0	0	0	0	200	0	0	500
	4	0	0	0	0	0	0	0	0	200	2	0	500
	5	0	0	0	0	0	0				8	10	500
	6	0	0	0	0	0	0				0	0	500
	7	0	0	0	0	0	0	0	2	500	2	5	200
	8	0	0	0	0	+	0	0	1	100	0	1	500
	9	0	0	0	0	0	0	0	0	200	0	10	500
	10	0	0	0	0	0	0	0	0	200	0	1	500
Controls	11	0	0	0	0	0	0	0	0	0	1	5	200
	12	0	0	0	0	0	0	0	0	0	0	0	500
	13	0	0	0	0	0	0						

the agglutinins for this corpuscle nor for the red corpuscles of the guinea pig.

c. Anti-rat experiment. - A third series of chicks with a genetic make up similar to those in the anti-rabbit group (exp. b) were treated with rat cells. Nine were given the injections and four kept for controls. Injections were made on the first, third, fifth and seventh days following hatching. Six days after the last treatment, their sera were tested for agglutinins with results as recorded in Table XII. It is evident that this treatment did not influence very decisively the appearance of the agglutinins for rat cells, although it is true that on the thirteenth day after hatching, the agglutinins for rat cells appeared in seven out of nine treated birds and none in the controls. It must be remembered that these agglutinins begin to appear normally about three days later than this. Hence little significance can be attached to this slight advance in time.

The results of the anti-guinea pig experiment are directly opposed to those of the anti-rabbit and anti-rat experiments; the appearance of the agglutinins being incited by the injection of guinea pig corpuscles but not by the cells of the rabbit or the rat. The question was raised as to the possibil-

Table XII. This table shows the effect of injections of chicks with the red corpuscles of the rat with respect to the time of origin of the hemagglutinins.

Method Used - Undiluted Serum							Protocol B
Age in days		13			23		
Kind of cell used		Guinea Pig	Rabbit	Rat	Guinea Pig	Rabbit	Rat
	No. of chicks						
CHICKS PREATED	1	0 0	0 0	0 0	0	0	500
	2	0 0	0 0	+ 0	0	0	500
	3	0 0	0 +	+ +	0	0	200
	4	0 0	0 0	+ +			
	5	0 0	0 0	+ +	0	0	500
	6	0 0	0 0	0 +	0	0	200
	7	0 0	0 0	+ +	0	0	200
	8	0 0	+ +	+ +	0	0	500
	9	0 0	0 0	0 0	0	0	500
CONTROLS	10	0 0	0 +	0 0	0	0	200
	11	0 0	0 0	0 0	0	0	200
	12	0 0	0 0	0 0	0	0	500
	13	0 0	0 0	0 0	0	0	

ity of the variety of chick used having influenced these results, since in the last two experiments chicks of genetically different make-ups were employed; the anti-guinea pig chicks were all of pure stock, while the anti-rabbit and anti-rat chicks were of a mixed variety.

To answer this question the first two experiments were repeated, this time using thirteen chicks of a mixed variety. These were divided into two lots, six receiving a series of injections with rabbit corpuscles and seven receiving a series of injections with guinea pig corpuscles. Treatments were begun on the second day after hatching and repeated on the fifth and seventh days. Cells were used in the same amounts and concentrations as in previous experiments. The first test was made when the birds were thirteen days old. The results are recorded in Tables XIII and XIV. We find here the same thing happening as in the previously injected birds. The guinea pig cells have stimulated their specific agglutinins to appear seventeen days earlier than normally, while the rabbit corpuscles failed to call forth specific antibodies.

While the agglutinating titre in the case of the anti-guinea pig series did not reach that which had been reached in the first series, nevertheless they are sufficiently high to be quite conclusive. The evidence presented by these two tests

Table XIII. This table shows the effect of injecting chicks (mixed variety) with red corpuscles of rabbits with respect to time of origin of hemagglutins.

Method Used	Undiluted Serum		
Age of chick in days	13		
Kind of cell used	Guinea Pig	Rabbit	Rat
1	0	+	0
	0	+	0
2	0	0	0
	0	0	0
3	0	0	0
	0	0	0
4	0	0	0
	0	0	0
5	0	0	0
	0	0	0
6	0	0	0
	0	0	0

Table XIV. This table shows the effect of injecting chicks (mixed variety) with guinea pig red corpuscles with respect to time of origin of hemagglutinins.

Method Used	Protocol B		
Age of chick in days	13		
Kind of cell used	Guinea pig	Rabbit	Rat
1	50	0	0
	50	0	0
2	20	0	0
	10	0	0
3	10	0	+
	5	0	0
4	5	0	0
	5	0	
5	10	0	0
	10	0	0
6	10	0	0
	10	0	0
7	100	+	+
	100	0	+

shows that the difference in results obtained from the injections of these two antigens is due, not to a difference in the chick, but to the kind of corpuscles used as antigen.

Since it has been shown that the anti-rabbit and anti-rat hemagglutinins do not appear until about the sixteenth day after hatching, it is puzzling to know why these antibodies do not respond to the injection of their specific antigens as the anti-guinea pig antibodies do. The following tentative explanation is offered: It has been demonstrated that rabbit corpuscles treated with chicken serum are rendered non-antigenic (14). This fact has been offered as an explanation for the failure to raise the hemolytic titre in adult fowls. Should it be true that hemolysins appear earlier than the hemagglutinins, and are present at the time when immunization with the rabbit and rat cells was begun in the chicks, it is possible that these cells are rendered non-antigenic for the production of their hemagglutinins.

6. The Normal and Immune Hemagglutinins in Chicken Serum not Identical.

When the serum from the adult chicken has been adsorbed with the red corpuscles of the rabbit, subsequent titrations made against the red corpuscles of the guinea pig and the

rabbit show that all the agglutinins for these two kinds of cells have been removed (part 3). A very significant difference in result was shown when this same experiment was repeated, substituting for the adult serum, serum from chicks which had been immunized against red corpuscles of the guinea pig (part 5). In this immune serum, pooled from four chicks, agglutinins active for guinea pig corpuscles were demonstrated in titre of 1-20, while those active for rabbit corpuscles could be demonstrated only in the undiluted serum. These adsorptions and titrations were carried out for these two kinds of cells by the same method as was used in part 3. The results are recorded in Table XV.

This experiment was repeated with practically the same results as the ones just described. Guinea pig corpuscles removed all of the agglutinins for guinea pig cells as was the expectation. The same holds for rabbit cells, but in this case, contrary to what happens with the normal serum, rabbit corpuscles failed to remove the agglutinins for the red cells of the guinea pig. This reaction of anti-guinea pig agglutinins toward the red corpuscles of the rabbit is interesting in that it shows the agglutinins in the immune serum which agglutinate guinea pig corpuscles are different

Table XV This table shows the results when immune anti-guinea pig serum is adsorbed with the red corpuscles of the guinea pig and rabbit and subsequently titrated against the red corpuscles from the same species.

Kind of cell used in adsorbing	Guinea Pig		Rabbit	
Kind of cell used in titrating	Guinea Pig	Rabbit	Guinea Pig	Rabbit
Titre before adsorbing	20	1	20	1
Titre after adsorbing	0	0	20	0

from the agglutinins in the normal serum which agglutinate guinea pig corpuscles. In other words, the latter have an affinity for the red corpuscles of the rabbit while the former do not.

DISCUSSION AND CONCLUSIONS

The differences in the amount of hemolysins and hemagglutinins present in chicken serum for the red corpuscles of other animal species bear no relation to the nearness or remoteness of kinship between the kind of species from which the cells are tested. There seems to be no a priori reason why the red cells of the rabbit should be five times as susceptible to these natural antibodies as are the red corpuscles of the guinea pig; or, on the other hand, why guinea pig cells and frog cells should be equally susceptible. That these natural antibodies act upon all but one of the fourteen kinds of corpuscles tested is a most striking fact.

Adsorbing the natural hemagglutinins with five different kinds of red cells has shown that the specificity of the antibodies concerned is relative and not absolute. Such specificity as there is depends upon the kind of cells used in the test. According to these results, of the four species tested, only a part of the anti-rabbit and a part of the anti-rat hemagglutinins are able to resist adsorption by other corpuscles. There remains,

however, the possibility that even these two can be adsorbed by cells other than those used in this test. Judging from the fact that guinea pig, sheep and goat corpuscles are able to adsorb hemagglutinins which do not effect their agglutination, the question of the specificity of the antibodies here studied is not yet settled.

The fact that the various hemagglutinins naturally arise in the blood stream of chickens at a definite time after hatching arouses some interesting speculation as to the cause of their appearance. The question as to why natural antibodies in general are present in the blood stream of animals is an old one. It is sometimes stated in textbooks (15) that the presence of natural anti-bacterial antibodies may be the response to the stimulus produced by the very small numbers of specific organisms which sometimes enter the blood stream by way of lymphoid tissue, such as Peyers patches, tonsils, etc. (16). Zingher (17), in performing Schick tests on 52,000 school children of New York City, found that the proportion of negative reactions was greater among those children living in tenement districts than among those living in the wealthier localities. He concluded that the so-called "natural immunity" which these children possessed for diphtheria toxin was in reality acquired immunity and that the greater percentage of those children having negative reactions occurred in the tenement sections

because these children had greater opportunities for exposure. It is difficult to believe that such an explanation can account for the appearance of hemagglutinins in the blood stream of the chick, for it is inconceivable that the red corpuscles of other animal species can enter the living body of the chick at this time. The nature of the development of the chick rules out the possibility that these antibodies can be transferred from the mother through the placenta (7). The work of Happ (3) on the time of the appearance of iso-hemagglutinins in man, together with the work of von Dungern and Ottenberg (5) showing that these affinities of the agglutinins are determined in the germ plasm would lead us to conclude that we must look for a more general physiological process underlying the development of the natural hemagglutinins in the blood stream of the chicken.

The response of the chick in producing specific antibodies following the injections of guinea pig corpuscles was not contrary to expectation. It is to be recalled that injecting adult fowls with red corpuscles does not produce any marked increase of antibodies active for these cells (14). This was explained on the ground that the blood of these chickens digested the cells to a point at which they were no longer antigenic. In the case of the chicks no lytic substances had yet appeared in the blood stream during the process of immunization and for this reason

it is tentatively concluded that the cells were able to act antigenically. More data are necessary, however, before such a conclusion can be held valid. The failure of chicks to respond to the injections of rabbit and rat cells is difficult to explain. It is suggested, however, that the early appearance of the natural antibodies active for these cells bears some relation to the case. Immunizing the chicks with the corpuscles of goat and sheep has shown that these cells have antigenic properties in common. This result corresponds to that obtained when these cells are injected into rabbits (18).

The difference between the normal and the immune anti-guinea pig hemagglutinins, when tested by adsorption, is definite. One objection might be raised regarding the normal antibody employed in this test. Since all the natural anti-guinea pig agglutinins are adsorbed by the red cells of the rabbit and the rat, one might justly ask whether these are in fact anti-guinea pig antibodies. It might be said that they are antibodies which act on other cells, as well as those of the guinea pig. On the other hand, there is evidence which supports the conclusion that this antibody is not identical with the others mentioned above. In the first place, the agglutinins active for guinea pig cells appeared at a time different from those active for the rabbit corpuscles. This means that the two must be different in at

least one respect. In the second place, the second adsorption of serum with fresh guinea pig cells fails to remove any additional anti-rabbit antibodies beyond those removed at first. These facts are sufficient to justify calling the natural antibody an anti-guinea pig hemagglutinin. That the normal and the immune anti-body here concerned are different, there can be no doubt. Therefore, it cannot be said that the immune anti-guinea pig hemagglutinins are a quantitative increase of the normal antibody already present.

SUMMARY.

1. The red corpuscles of thirteen out of fourteen animal species here tested were found to be agglutinated by pooled serum from adult chickens. Twelve species of corpuscles were also hemolysed; the red cells of the pigeon and the goat being the exceptions. Goat cells were neither hemolysed nor agglutinated by this serum.

2. Iso-hemagglutinins could not be demonstrated in the active and inactive chicken serum.

3. The specificity of the hemagglutinins in pooled normal adult chicken serum was shown to be relative and not absolute when tested by specific adsorption with red corpuscles of the rabbit, rat, guinea pig, sheep and goat.

4. The time of the appearance of the hemagglutinins in the

blood stream of the chicken is as follows:

a. Anti-guinea pig: About 30 days after hatching.

b. Anti-rabbit:..... About 16 days after hatching.

c. Anti-rat:..... About 16 days after hatching.

5. Immune anti-guinea pig hemagglutinins were stimulated to appear fifteen days earlier than the normal antibodies by intraperitoneal injections of the specific antigens. Anti-rabbit and anti-rat hemagglutinins were not stimulated to appear any earlier than in the normal by the injections of their specific antigens.

6. Normal anti-guinea pig and immune anti-guinea pig hemagglutinins show one difference in reaction, the normal antibody being adsorbed by rabbit's red corpuscles while the immune antibody is not.

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BIOGRAPHY

Clenmie Elsie Bailey was born August 18, 1892, in Vigo County, Indiana. She attended the public schools of this county and graduated in 1911 from the High School at Prairie Creek, Indiana. In 1911 she entered the Indiana State Normal School, at Terre Haute, Indiana. The four years spent here were not consecutive as she taught three school years in the grades during this time. She received her A.B. degree, with Biology as a major, in June 1917, from this institution. During the school year of 1917-1918 she taught Science in the Fairbanks High School Sullivan County, Indiana. From there she went to Manhattan, Kansas, to become assistant in Genetics in the Department of Zoology ir the Kansas State Agricultural College. In the fall of 1918 she became acting head of the Department of Physiology and Zoology in the Indiana State Normal School, Eastern Division, at Muncie, Indiana. In October, 1919, she entered the Johns Hopkins University, School of Hygiene and Public Health, as a candidate for the degree of Doctor of Science in Hygiene. During this time her major work was done in the Department of Immunology. Since October, 1922 she has been assistant director of the University Pathological Laboratory at the Medical School, Little Rock, Ark.